REMARKS

Status

Claims 15-17, 25, and 29-34 are pending and under examination.

Drawings

Formal drawings are submitted herewith.

Rejection of Claims 15-17 and 29-34 Under Section 103

Claims 15-17, 25 and 29-34 were rejected as allegedly obvious in view of U.S. Pat. No. 6,355,459 ("Schupp"), U.S. Pat. No. 6,391,594 ("Khosla A") and WO 97/02358 ("Khosla B"). Applicants respectfully traverse.

The Office acknowledges that Schupp does not teach the chemical compound epothilone D "by name" and contains errors concerning the synthesis of epothilone D. The Examiner asserts that Schupp nonetheless teaches an "epothilone derivative of epothilone D, *i.e.*, epothilone A and epothilone B."

As Applicants understand it, the position of the Office is premised on the belief that "one of ordinary skill in the art would consider both epothilone A and epothilone B derivatives of epothilone D" (Office Action page 4). Applicants respectfully disagree. For example, the specification (page 18, lines 5-9) explains that "As used herein, the phrase "epothilone derivative" refers to a compound that is produced by a recombinant epothilone PKS in which at least one domain has been either rendered inactive, mutated to alter its catalytic function, or replaced by a domain with a different function or in which a domain has been inserted." Taken together with the rest of the specification, which is in one aspect directed to production of novel epothilones with improved properties (e.g., less cytotoxicity) compared to naturally occurring epothilones A and B, it would be clear to the ordinarily skilled practitioner that epothilones A and B are not encompassed in the recitation of "epothilone D derivatives." For further clarity, and to expedite prosecution, Applicants have amended the claims to recite that the epothilone D derivative comprises "a double bond between C-12 and C-13." Support for this amendment is replete in the specification. For example, at page 33, line 23, the specification explains that the C-12-C-13 alkene is a distinguishing

characteristic of "epothilone D (*or an epothilone D derivative*)" [emphasis added]. As a further example, at page 16, line 22, the specification explains that epothilones C and D differ from epothilones A and B because "they lack the C-13 hydroxyl and have a double bond between C-12 and C-13."

The Office also refers to Examples 13 and 14 of the Schupp reference and asserts "The fact that epothilone B is made under the fermentation conditions of Schupp indicates that epothilone D must have been formed during the fermentation." Applicants respectfully submit that this basis for rejection is both factually and legally deficient. First, the instant claims are directed to production of epothilones by a non-*S. cellulosum* cell. Schupp Examples 13 and 14 do not show that epothilone B is made in a non-cellulosum cell, as recited in the present claims. The examples show the production and isolation of epothilones A and B from the native *Sorangium cellulosum* producer. The Schupp reference did not demonstrate production of any products, and in fact taught away from the expectation epothilones C and D would be produced by proposing that the product of the PKS genes is the 13-alcohol.

This is important for a number of reasons. The combination of references cited by the Examiner would not have led the ordinarily skilled artisan to believe that epothilone D derivatives could be produced in non-*Sorangium* heterologous host cells using an epothilone PKS with a modified *epoE* gene. Instead, such artisans would have believed that it was impossible to make epothilone B (much less epothilone D) in a heterologous host using the PKS genes described by the Schupp reference, because, *inter alia*, the Schupp reference teaches that a non-epothilone gene cluster methyltransferase gene is required to place the C-12 methyl in epothilone B and does not even mention epothilone D (which Applicants teach is a precursor of epothilone B). In short, the combination of references relied on by the Office fails *two* arms of obviousness test: taken together they do not render the present claims obvious because they fail to suggest the present invention *and* because they fail to provide the practitioner with any expectation of success. Each of these deficiencies is independently sufficient to overcome the rejection.

Further, since the Office acknowledges the Schupp reference did not teach the chemical compound epothilone D, this rejection appears to be a rejection based on inherency (i.e., that

epothilone D must have been made, albeit not appreciated).[1] Notwithstanding the deficiencies of the Schupp reference, as explained above, it is settled law that that, while a single prior art reference may anticipate because of what it inherently discloses or embodies, an inherent feature may be relied upon to establish obviousness, if at all, only if the inherency *would have been* obvious to one of ordinary skill in the art. (See e.g., *Kloster Speedsteel AB v. Crucible Inc.*, 230 USPQ 81, 88 Fed. Cir. 1986.) Clearly, it would not have been obvious to one of ordinary skill reading the Schupp reference that epothilone D would be produced by *any* cell, since Schupp did not describe epothilone D.

Rejection of Claim 25 Under Section 103

Claim 25, an independent claim, was included in the rejection under Section 103 articulated in the Office Action. However, the Office did not provide any reasoning to support the rejection of clam 25. The Office acknowledges the Schupp reference "contains some errors regarding some chemical steps." Applicants understand these errors include the teaching by the Schupp reference that an exogenous (non-cluster) gene product (a methyltransferase) is required to form epothilone B. Phrased differently, Schupp did not recognize the MT domain of module 8. Applicants submit it is beyond dispute that, considering this, the cited references could not have made the subject matter of claim 25 obvious.

Rejections Under Section 112, Second Paragraph

Claims 15 and 29, from which claims 16-17 and 30-34 depend, stand rejected as allegedly indefinite. The Office asserts that claims 15 and 29 are indefinite and confusing because they refer to "a modified epothilone synthase, i.e., a singular protein." The Examiner states that from reading the specification at least five polyketide synthases are required for the synthesis of the macrolactone.

The instant specification teaches that "The epothilone PKS [singular] is a multiprotein complex composed of the gene products of the *epoA*, *epoB*, *epoC*, *epoD*, *epoE*, and *epoF* genes"

[1] The Office does not state that the rejection is based on inherancy. However, to thte extent this is implied, Applicants provide this response.

(page 16, lines 1-2) and that "multiple proteins . . . constitute the PKS [singular]" (page 16, line 10). This usage is consistent with the usage in the claims. It is also consistent with usage in the scientific literature. A copy of a publication coauthored by one of the inventors of the present application is enclosed to illustrate this point (Julien and Shah, 2002, "Heterologous Expression of Epothilone Biosynthetic Genes in *Myxococcus xanthus*" *Antimicrobial Agents and Chemotherapy* 46:2772-78; see e.g., page 2772, col. 2, beginning of first full paragraph) but many other publications could be cited. Thus, although alternative terminologies may be used, Applicants' terminology is conventional (and clearly within the latitude permitted an applicant to be "his own lexicographer"). Thus, Applicants submit the terminology is acceptable and the claims clear. Indeed, it is possible the claims might be *less* clear if amended to use terminology preferred by the Office. To expedite prosecution Applicants would consider any specific language suggested by the Examiner. Thus, if the Examiner continues to believe that an adjustment of the claim language is desirable, he is respectfully invited to contact the undersigned Applicants' representative to discuss this.

Applicants also note that claim 29, as previously amended, refers to the gene products of the epoA, epoB, epoC, epoD, and epoF genes. Applicants believe claim 29 is clear.

Rejections Under Section 112, First Paragraph

Claims 15-18 and 29-34 were rejected because the Office states that only the *epoE* gene of *Sorangium cellulosm* is described in the specification. This rejection was made previously, in the Office Action dated May 22, 2003, but Applicants inadvertently failed to discuss the rejection in the response filed October 22, 2003. Applicants thank the Examiner for drawing Applicants' attention to this omission.

Claim 15 has been amended to specify that the epothilone PKS is a *Sorangium cellulosum* PKS and believe this amendment overcomes the rejection. Applicants note that, although not specifically discussed in the prior response, claim 29 was previously amended to recite that the PKS comprises "the proteins encoded by the *Sorangium cellulosum epoA*, *epoB*, *epoC*, *epoD*, and *epoF* genes." Therefore, Applicants believe claims 15 and 29, and the claims dependent from them, are in compliance with the requirements of Section 112, first paragraph.

Finality of the Last Office Action

The present rejection under Section 112, second paragraph, is a new rejection. It is not apparent how this rejection (for example, of claim 15) was necessitated by an amendment made by the Applicants. Accordingly, Applicants respectfully submit the finality of the Office Action should be withdrawn.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 300622003111. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

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Heterologous Expression of Epothilone Biosynthetic Genes in *Myxococcus xanthus*

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Epothilones are potential anticancer drugs that stabilize microtubules in a manner similar to paclitaxel (Taxol). Epothilones are produced from the myxobacterium *Sorangium cellulosum*, which has a 16-h doubling time and produces only milligram-per-liter amounts of epothilone A and epothilone B. Furthermore, genetic manipulation of *S. cellulosum* is difficult. To produce epothilones in a more genetically amenable and rapidly growing host, we chose the closely related and best-characterized myxobacteria *Myxococcus xanthus*. We inserted 65.4 kb of *S. cellulosum* DNA that encompassed the entire epothilone gene cluster into the chromosome of *M. xanthus* by a series of homologous recombination events. The resulting strain produced epothilones A and B. Construction of a strain that contained a mutation in *epoK*, the P450 epoxidase, resulted in production of epothilones C and D.

Epothilones are potent cytotoxic macrocyclic lactones that show promise as anticancer drugs (21, 22, 26). The mechanism of action is similar to the anticancer drug paclitaxel (Taxol); both bind and stabilize microtubules, which leads to cell death (3). Epothilones have superior features relative to paclitaxel. First, epothilones are more water soluble. This may enable a formulation without the use of the solubilizing agent cremophor, currently used in formulations of paclitaxel. Cremophor, on its own, can affect cardiac function and cause severe hypersensitivity (25). Second, epothilones are effective against tumors resistant to paclitaxel (3, 31). These advantages make epothilones likely successors to paclitaxel.

The need for sufficient material is a major obstacle to the development of epothilones as marketable drugs. The total synthesis of epothilone A and epothilone B has been accomplished (2, 32). However, the number of steps required for synthesis of these molecules precludes this as an economical method of production. Fermentation methods thus remain the favored route of production.

Epothilones are produced from the gram-negative myxobacterium *Sorangium cellulosum* (8). The reported yields of epothilones from *S. cellulosum* strain So ce90 are approximately 20 mg of epothilone A per liter and 10 mg of epothilone B per liter (8). A disadvantage of *S. cellulosum* is the relatively long doubling time, which is approximately 16 h and is the longest of all myxobacteria. Furthermore, *S. cellulosum* is difficult to engineer, due to the low efficiency of introducing DNA into the bacterium (11) and the limited number of molecular tools and markers that have been developed.

Recently, the epothilone biosynthetic gene cluster was sequenced, and the genes were introduced into *Streptomyces coelicolor*, a common host used for production of a variety of polyketides from actinomycetes (33). The heterologous strain produced small quantities of epothilones; production of epothilones in *S. coelicolor* may have a cytotoxic effect (L. Tang et al., unpublished data). Thus, an alternative heterologous host is desired.

We speculated that a superior expression host for epothilones might be another myxobacterium and chose *Myxococcus xanthus* for several reasons. First, *M. xanthus* is the best-characterized myxobacterium and is readily amenable to engineering (35). Second, *M. xanthus* has a significantly shorter doubling time than *S. cellulosum* (5 versus 16 h), which would in itself enhance volumetric productivity. Third, expression of the epothilone genes in *M. xanthus* may not require the engineering of new promoters, because *M. xanthus* and *S. cellulosum* are closely related organisms.

The epothilones are polyketides that are synthesized by a type I polyketide synthase (Fig. 1). These enzymes are large multifunctional complexes organized in a modular fashion and catalyze the successive condensation of carboxylic acid residues from their coenzyme A (CoA) esters, typically malonyl-CoA and methylmalonyl-CoA (10). Polyketide synthase enzymes are synthesized as an apo form and are converted to the holo form by the addition of a phosphopantetheinyl (P-pant) moiety to a serine residue of the acyl or peptidyl carrier protein (ACP or PCP) domains by an enzyme called P-pant transferase (18). Thus, a heterologous host must synthesize malonyl-CoA and methylmalonyl-CoA as well as harbor a P-pant transferase.

Before initiating this work, it was unknown whether *M. xanthus* contains a P-pant transferase or methylmalonyl-CoA. It is known that strains of *M. xanthus* produce the polyketide myxovirescins, also known as TA. Thus, it is likely to contain a P-pant transferase, but whether it would function on the ACP and PCP domains of the epothilone polyketide synthase remained to be determined. Although myxovirescins contains methyl groups extending from the ring, they are all derived from *S*-adenosylmethionine and not from the utilization of methylmalonyl-CoA. Thus, evidence for the production of methylmalonyl-CoA cannot be deduced from the polyketides that are made naturally from this host. Work studying the developmental life cycle of *M. xanthus* has revealed the presence of the genes for propionyl-CoA carboxylase (16, 17), an enzyme used to synthesize methyl malonyl-CoA. Thus, *M. xan-*

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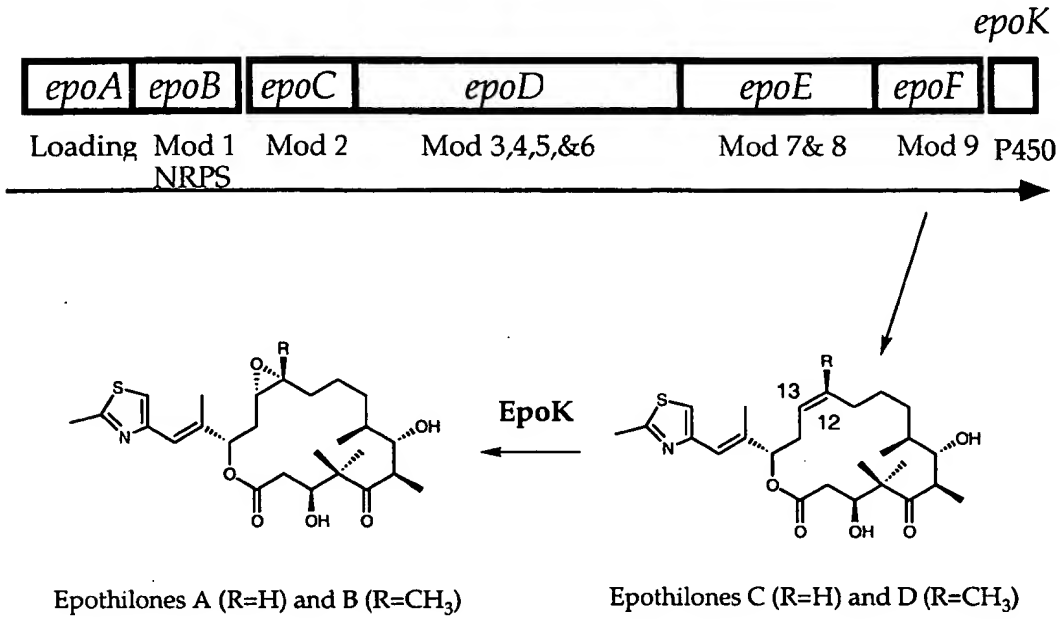

FIG. 1. Genetic map of the epothilone biosynthetic gene cluster. The boxes represent genes, and the modules encoded by each are designated below. The long arrow represents the direction of transcription.

thus is likely to possess required components to synthesize epothilone.

Because of the advantages of using *M. xanthus* as a host for production of epothilone, we introduced the epothilone genes from *S. cellulosum* into the chromosome of *M. xanthus*. We demonstrate that the engineered strain produces epothilones. We also constructed mutations in *epoK*, the CYP450, which is responsible for the formation of the epoxide at C-12–C-13. This strain produces epothilone C and epothilone D.

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* strains XL1-Blue and DH10B were used for transformations. *M. xanthus* strain DZ1 was the recipient for the epothilone genes (36). It is streptomycin resistant and is a nonmotile strain that is unable to form fruiting bodies

Media and growth conditions. *E. coli* with plasmids were grown in Luria-Bertani medium containing 0.5% NaCl at 37°C supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml).

DZ1 was grown in CYE (4) at 30 or 32°C. For selection of galactose resistance in *M. xanthus*, cells were plated in 2.5 ml of CYE top agar and poured onto CYE plates containing 1% galactose. The following antibiotics were used for *M. xanthus*: kanamycin (50 μg/ml) and oxytetracycline (10 to 15 μg/ml).

To test for the production of epothilone, cells were cultured in CMM (0.5% Casitone, 0.2% $MgSO_4$, 10 mM morpholinepropanesulfonic acid [MOPS] [pH 7.6]) supplemented with the following amino acids (1 mg/liter): serine, glycine, and alanine. The cells were initially grown in CYE medium, and while in mid-log phase a 5% inoculum was used to inoculate a flask containing production medium. The cultures were grown at 30°C for 64 to 72 h.

XAD-16 is an absorber resin that was used in production cultures to bind the epothilones. To prepare the XAD-16, 2% was added to a fermentation flask, covered with water, and autoclaved. Afterwards, the water was removed, CMM was added, and the culture was inoculated.

Extraction of epothilones. To extract epothilones from cells and from the culture (cells and medium), an equal volume of acetone was added and shaken at room temperature for an hour. The acetone mixture was extracted twice with an equal volume of ethyl acetate, and the ethyl acetate fraction was dried and resuspended in acetonitrile.

To analyze epothilones bound to XAD-16, the culture broth was removed and the resin was washed twice with 5 volumes of MilliQ water. The epothilones were eluted with methanol and analyzed by liquid chromatography-mass spectrometry (33).

DNA methods. Standard DNA protocols were used for *E. coli*. The isolation of *M. xanthus* chromosomal DNA was done as previously described (1). Electroporation of *M. xanthus* was described by Kashefi and Hartzell (15)

Transposition reactions. The tetracycline resistance gene was added to the cosmids pKOS35-70.1A2 and pKOS35-79.85 (13) using the in vivo transposition reaction kit from Epicentre Technologies. The transposon used was EZ::TN<TET-1>, and the reaction was performed as recommended by the manufacturer. Resulting tetracycline-resistant colonies were screened for resistance to kanamycin. Those colonies resistant to tetracycline and sensitive to kanamycin were kept, and the DNA was digested with restriction enzymes to verify that no deletions had occurred. The tetracycline resistant versions of pKOS35-70.1A2 and pKOS35-79.85 are pKOS90-38 and pKOS90-23, respectively.

Construction of pKOS35-154 and pKOS90-22. Plasmids that contained regions of the *epo* genes flanked by *M. xanthus* DNA were constructed for homologous recombination of the *epo* genes into the chromosome. Plasmid pKOS35-154 was constructed in several steps. First, the ca. 3-kb *Bam*HI-to-*Nde*I fragment from KG2 (35) was ligated into the *Bgl*II and *Nde*I sites of pSL1190 (Amersham-Pharmacia). This plasmid, pKOS55-178, contains the kanamycin resistance and *galK* cassette that is used for positive and negative selection in *M. xanthus*. Next, a 4.7-kb *Not*I fragment from cosmid pKOS35-79.85, containing a portion of the coding sequence for module 7, was ligated into the *Not*I site of pKOS55-178 to create pKOS55-183.b. Plasmid pKOS55-183.b was cleaved with *Mfe*I, and the DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated with a 5,088-bp *Srf*I fragment from pKOS35-79.85. This plasmid, pKOS90-1, contains two regions of the *epo* gene cluster flanking the kanamycin resistance and *galK* cassette. To add flanking DNA from *M. xanthus* to recombine the *epo* genes from pKOS90-1 into the chromosome, plasmid pKOS35-151 was constructed as follows. Plasmid pBJ130 (12), which carries the *dev* locus from *M. xanthus*, was cleaved with *Kpn*I; the DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated with the polylinker from pSL1190 (Pharmacia), which had been cleaved with *Eco*RI and *Hin*dIII; and the DNA ends were made blunt with the Klenow fragment. This plasmid, pKOS35-151, was cleaved with *Spe*I and *Eco*RI and ligated with the 12-kb *Spe*I-*Eco*RI fragment from pKOS90-1 to create pKOS35-154.

Plasmid pKOS90-22 was used for recombining the 5′ end of the *epo* gene cluster into the *M. xanthus* chromosome. First, a portion of the *epo* genes

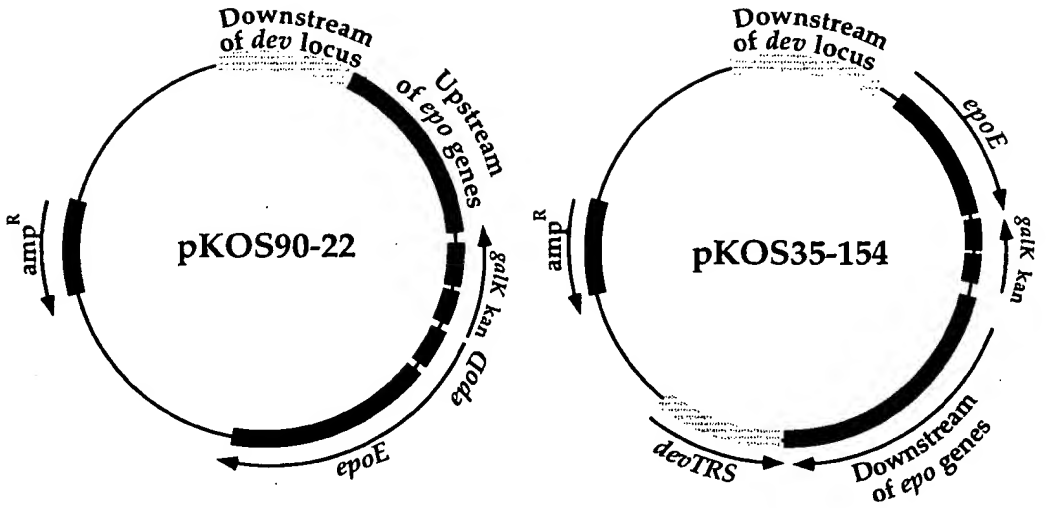

FIG. 2. Plasmids used for introduction of recombination sites for epothilone genes. Lightly shaded boxes represent regions of DNA from *M. xanthus*. Dark boxes represent selectable markers or regions of DNA from the epothilone gene cluster. Arrows represent the direction of transcription.

encoded by module 6 and module 7 was isolated as an 8.8-kb *Bgl*II fragment from cosmid pKOS35-70.4 (13) and ligated into the *Bam*HI site of pKOS55-178 to create pKOS90-12. To isolate a fragment upstream of the start of the *epo* genes, a 9.5-kb *Nsi*I-to-*Avr*II fragment from cosmid pKOS35-70.8A3 (13) was ligated into the *Pst*I and *Avr*II sites of pSL1190 to yield pKOS90-13. Next, pKOS35-151 was cleaved with *Eco*RI and *Hin*dIII, the DNA ends were made blunt with the Klenow fragment, and the plasmid was religated. This plasmid, pKOS90-5, was cleaved with *Mfe*I and *Avr*II and ligated with the 11.5-kb *Eco*RI-*Spe*I fragment from pKOS90-12 to construct pKOS90-17. Finally, pKOS90-22 was constructed by cleaving pKOS90-17 with *Spe*I and ligating it with the 9.5-kb *Spe*I-*Avr*II fragment from pKOS90-13.

Construction of an *epoK* mutant. To construct an *epoK* mutant, a kanamycin resistance cassette was inserted into the *epoK* gene. This was done by isolating the 4,879-bp fragment from pKOS35-79.85, which contains *epoK*, and ligating it into the *Not*I site of pBluescript SKII(+). This plasmid, pKOS35-83.5, was partially cleaved with *Sca*I, and the 7.4-kb fragment was ligated with the 1.5-kb *Eco*RI-*Bam*HI kanamycin resistance gene from pBJ180-2 (B. Julien and D. Kaiser, unpublished), which had the DNA ends made blunt with the Klenow fragment, to yield plasmid pKOS90-55. Finally, the ca. 400-bp RP4 *oriT* fragment from pBJ183 (Julien and Kaiser, unpublished) was ligated into the *Xba*I and *Eco*RI sites to create pKOS90-63. This plasmid was linearized with *Dra*I and electroporated into the *M. xanthus* strain K111-32.25 to create K111-40.

To create a markerless *epoK* mutation, pKOS35-83.5 was cleaved with *Sca*I, and the 2.9- and 4.3-kb fragments were ligated together. This plasmid, pKOS90-101, has an in-frame deletion in *epoK*. Next, the 3-kb *Bam*HI-*Nde*I fragment from KG2, which had the DNA ends made blunt with the Klenow fragment and contains the kanamycin resistance and *galK* genes, was ligated into the *Dra*I site of pKOS90-101 to create pKOS90-105. This plasmid was electroporated into K111-32, and kanamycin-resistant colonies were selected. To replace the wild-type copy of *epoK* with the deletion, the second recombination event was selected by growth on galactose plates. These galactose-resistant colonies were screened for production of epothilone C and D. This strain is designated K111-72.

RESULTS

Construction of an *M. xanthus* strain containing the epothilone gene cluster. To reconstruct the epothilone gene cluster in *M. xanthus*, we chose to introduce the cluster into the chromosome by homologous recombination. This entails placing regions of homology from the epothilone genes into the chromosome of *M. xanthus* and using these regions as recombination sites for cosmids containing parts of the *epo* gene cluster. The location on the *M. xanthus* chromosome chosen for the recombination site was 3′ of the *dev* operon (34). This region encodes no recognizable genes, and insertions in this region are not predicted to disrupt any transcription.

The plasmids used and the strategy for introducing part of the *epo* genes in *M. xanthus* are diagramed in Fig. 2 and 3. First, plasmid pKOS35-154 was constructed. This plasmid contains a 7-kb fragment of *M. xanthus* DNA, 2.8 kb of the *dev* operon and 4.2 kb downstream of *dev*, and two regions from the *epo* gene cluster: a region from module 7 and a region 4.7 kb downstream of *epoK*. Between the two *epo* fragments is the kanamycin resistance gene from Tn*5* and the *E. coli galK* gene. Plasmid pKOS35-154 was linearized and electroporated into *M. xanthus* to produce strain K35-159 (Fig. 3A). Kanamycin-resistant colonies arise from a double recombination event: one recombination with the *dev* locus and another with the region 3′ to *dev*. This introduces regions from the *epo* gene cluster that serve as recombination sites for a cosmid containing the downstream half of the *epo* genes. Next, the cosmid pKOS90-23, which contains *epoE*, *epoF*, *epoK*, and downstream DNA, was electroporated into K35-159 (Fig. 3B). This strain, K35-174.6, results from a single recombination event. To remove the cosmid vector and leave only the region between the two flanking *epo* regions inserted by plasmid pKOS35-154, a second recombination event was selected by isolating galactose-resistant colonies. The *E. coli galK* gene renders *M. xanthus* sensitive to 1% galactose and provides a convenient method for isolating bacteria that have lost the *galK* gene, which occurs by a second recombination event between the flanking *epo* region and its homologous DNA on the cosmid. This results in strain K35-175, which contains part of *epoE*, the complete *epoF* and *epoK* genes, and 4.7 kb of DNA downstream (Fig. 3C). The 4.7 kb of DNA downstream of *epoK* contains several putative open reading frames (arrows in Fig. 3C). None of the open reading frames contain homologies to proteins which would suggest a function. However, the first and last open reading frames encode proteins predicted to

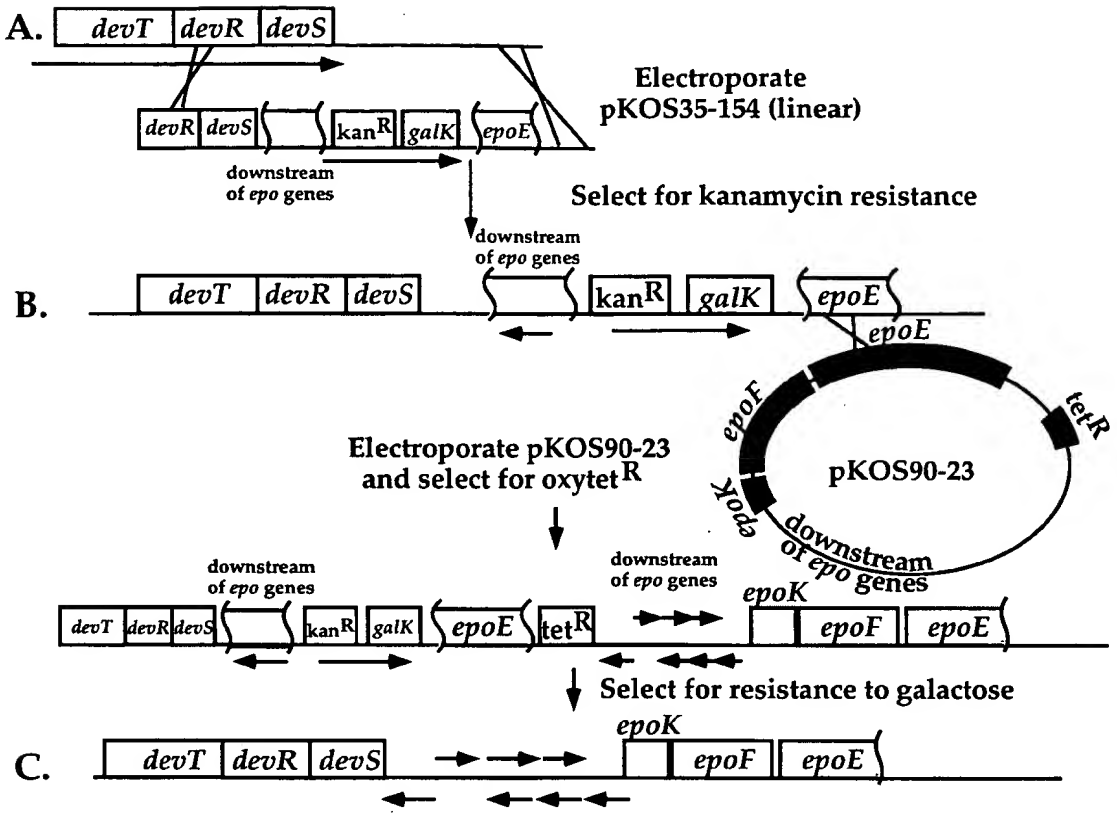

FIG. 3. Schematic for introduction of a portion of the epothilone genes into the chromosome of *M. xanthus*. (A) The introduction of recombination sites for the epothilone genes from pKOS35-154. Arrows indicate direction of transcription. Boxes with curved ends represent partial genes or fragments of DNA from *S. cellulosum*. (B) Introduction of the cosmid pKOS90-23 into the chromosome. (C) Resulting genetic structure after selecting for the second recombination event to remove duplicated regions and regions with the kanamycin resistance and *galK* genes. Arrows indicate putative open reading frames and their direction of transcription in the 4.5 kb of *S. cellulosum* DNA downstream of *epoK*.

harbor membrane spanning regions and so may code for transporter proteins.

To insert *epoA*, *epoB*, *epoC*, *epoD*, and the remaining region of *epoE*, a second round of recombination, similar to the first, was performed. Plasmid pKOS90-22 was linearized and electroporated into K35-175 to construct K111-13.2. Next, cosmid pKOS90-38 was electroporated into K111-13.2 to construct K111-13.22. Finally, to complete the construction of the gene cluster, a galactose-resistant version of K111-13.22 was selected. This final recombinant strain contains the epothilone gene cluster plus 4.7 kb upstream of the translational start of *epoA* and 4.7 kb downstream of the translational stop of *epoK*. Eighteen isolates were examined for epothilone production and analyzed by Southern blotting. From this, the two isolates that produced the highest amounts of epothilones were further examined and are referred to as K111-32 isolates 1 and 2.

Although the epothilones are secreted into the medium when produced in *S. cellulosum*, we were not certain whether a specific transporter was needed to transport the epothilones out of the cell. In constructing the *M. xanthus* epothilone producer, two open reading frames that appear to encode membrane-spanning proteins were contained within the 65.4 kb of DNA. The two isolates of K111-32 were grown in CMM medium for 60 to 72 h, the period of maximum epothilone accumulation (data not shown), and the levels of epothilone were analyzed in the total culture, which contains the cells and medium together; in the medium; and in the cells alone. The results (Table 1) show that the epothilones are secreted into the medium and only trace amounts are retained in the cells. We currently do not know if the two open reading frames, predicted to encode membrane-spanning proteins, are required for the export of the epothilones.

Comparison of the epothilones produced in *M. xanthus* versus those produced in *S. cellulosum* shows that the levels of epothilone B are about 100-fold lower in *M. xanthus* under the growth conditions tested. Interestingly, the ratio of epothilone A to epothilone B produced in *M. xanthus* is approximately 1:10, in stark contrast to *S. cellulosum*, where the ratio of

TABLE 1. Epothilone production in K111-32

Isolate no.	Epothilone production[a] (mg/liter) in:					
	Total cell culture		Broth		Cells	
	EpoA	EpoB	EpoA	EpoB	EpoA	EpoB
1	0.013	0.12	0.013	0.12	ND	D
2	0.017	0.16	0.012	0.12	ND	D

[a] Abbreviations: ND, none detected; D, detectable but below the level of quantitation.

epothilone A to epothilone B is roughly 2:1. The difference between epothilone A and epothilone B is the presence of a methyl group at carbon 12. This methyl group is derived from methylmalonyl-CoA and not by C methylation, which indicates that the acyltransferase from module 4 is able to accept either malonyl-CoA or methylmalonyl-CoA (9). Thus, in *M. xanthus*, there may be a larger pool of methylmalonyl-CoA during epothilone production that increases the production of epothilone B over epothilone A.

Construction of an *epoK* mutant. Epothilone A and epothilone B are the major constituents of the fermentation of the natural and heterologous hosts. However, epothilone D appears to have the best therapeutic index (5–7, 30). Of the two other candidate drugs currently in clinical trials (22, 26), epothilone B and aza-epothilone B, epothilone D proved superior in reducing tumor size in nude mice containing MX-1 tumor cells. Epothilone B is synthesized from epothilone D by the formation of the epoxide at C-12–C-13, which is added by EpoK, a cytochrome P450 (Fig. 1). This has been demonstrated in vitro by the conversion of epothilone D to epothilone B in the presence of purified EpoK (13). Thus, a strain that contains an *epoK* mutation would be a valuable strain for production of epothilone D.

Two strains that contain mutations in *epoK* were constructed. One contains an insertion of the kanamycin gene from Tn*5*, and the other has an in-frame deletion of *epoK* that removes 705 bp, or 56% of the gene. Although there are no genes downstream of *epoK* that are necessary for the synthesis of epothilones, there may be genes important for export, particularly the first open reading frame downstream of *epoK* that is predicted to contain several transmembrane spanning regions. Therefore, it was important to construct mutations that will not have polar effects. With the insertion, the kanamycin resistance gene is oriented such that its promoter would drive expression of genes downstream of *epoK*.

Strains harboring mutations in *epoK* were grown, and the amount of epothilones produced are presented in Fig. 4 A. Isolates of K111-40 contain the insertion of the kanamycin resistance gene (*epoK::npt*), whereas the isolates of K111-72 contain an in-frame deletion (Δ*epoK*). The data reveal that the levels of epothilone A from the isolates of K111-32 are similar to the levels of epothilone C from the strains carrying a mutation in *epoK* and that both types of mutations, the insertion and the in-frame deletion, result in the same levels of production. Interestingly, the levels of epothilone D produced are about fivefold lower than the *epoK* mutant strains relative to epothilone B produced from K111-32.

Because the resin XAD-16 is used in the fermentation of the *S. cellulosum* strain that produces epothilone, we investigated whether binding of the epothilones to the resin XAD-16 would sequester them from the cells and stabilize the compounds, particularly epothilone D. Figure 4B shows the results of fermentations in the presence XAD-16. The levels of epothilone D produced from the strains harboring the *epoK* mutation are higher in the presence of XAD and are equivalent to epothilone B levels produced in K111-32. These results suggest that epothilone D is unstable during the fermentation process relative to the other epothilones.

Interestingly, the strain K111-32 produced equal amounts of epothilone B and epothilone D in the presence of XAD-16,

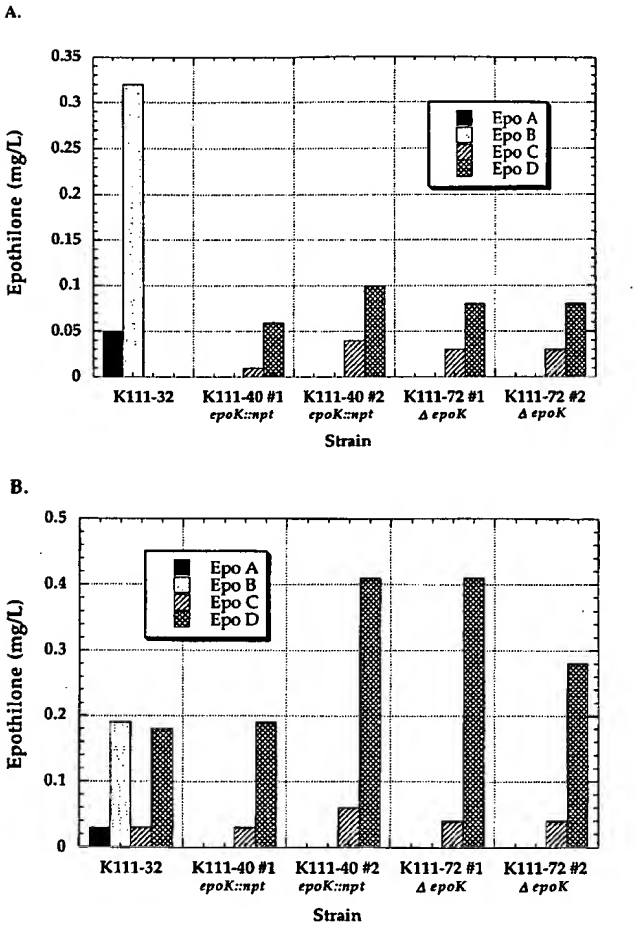

FIG. 4. Production levels from various strains in the absence (A) or presence (B) of XAD-16.

even though it contains a fully functional *epoK*. This is not seen in epothilone production from *S. cellulosum*. In the presence of XAD-16, epothilone C and epothilone D make up less than 10% of the total amount of epothilones produced in shake flask experiments (B. Julien, unpublished data). This suggests that in *S. cellulosum*, the majority of the epothilone D is converted to epothilone B before release into the medium, whereas in *M. xanthus*, epothilone D has an equal probability of being released into the medium as being acted upon by EpoK.

DISCUSSION

Myxobacteria produce a variety of novel polyketides with novel modes of action, including the epothilones (23, 24). Current understanding of polyketide synthase genes allows them to be engineered to produce novel compounds that may have superior activity relative to the natural ones. However, engineering of genes in strains of *S. cellulosum*, as well as in most other myxobacteria, would be extremely difficult, due to the lack of genetics and molecular techniques available. Although *Streptomyces* expression systems have been developed for production of polyketides (14), the yields can be low, and unfore-

seen side effects of heterologously producing a non-actinomycetes compound, particularly from a gram-negative bacterium, can be problematic, as seen with the epothilones (Tang et al., unpublished data). Thus, the work presented here demonstrates that *M. xanthus* provides an important alternative host for production of polyketides, especially those from other myxobacteria. Although the levels of epothilones reported here are low, through medium development and using a feeding strategy of Casitone and methyl oleate, current levels of epothilone B are higher than those obtained with the epothilone-producing *S. cellulosum* strain So ce90 (19). Furthermore, mutants that harbor a mutation in *epoK* produce 50 to 100 times more epothilone D than does So ce90.

From the work presented here, important information was obtained as to the use of *M. xanthus* as a heterologous host. First, the strain used has a P-pant transferase that is able to modify both ACP and PCP domains. Second, the strain has a pool of methylmalonyl-CoA during the time of epothilone synthesis. Furthermore, during this time period, there may be more methylmalonyl-CoA than malonyl-CoA, as evidenced by the ratios of epothilone A and epothilone B produced, which is approximately 1:10. Finally, the promoter that drives expression of the epothilone genes in *S. cellulosum* most likely is the same one that is utilized in *M. xanthus*. Since no specific regulator proteins have been found associated with any of the several polyketide synthase genes sequenced from *S. cellulosum* or *Stigmatella aurantiaca* (13, 20, 27–29), there is likely some global regulator that activates production of secondary metabolites at the onset of stationary phase, and this regulation is similar in many myxobacteria. Further support is provided by the fact that the myxothiazole promoter from *S. aurantiaca* can drive expression of the epothilone genes in *M. xanthus* (S. Shah and B. Julien, unpublished data).

Now that epothilone can be produced in a heterologous host tolerant of its production and amenable to genetic manipulation, the stage is set to make new compounds that can only be made by engineering of the genes and to identify those with increased therapeutic value.

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